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Applicant KAWAI, Shinji et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

22 November 1999 (22.11.99)

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(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

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Date of mailing (day/month/year)
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IMPORTANT NOTIFICATION

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1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

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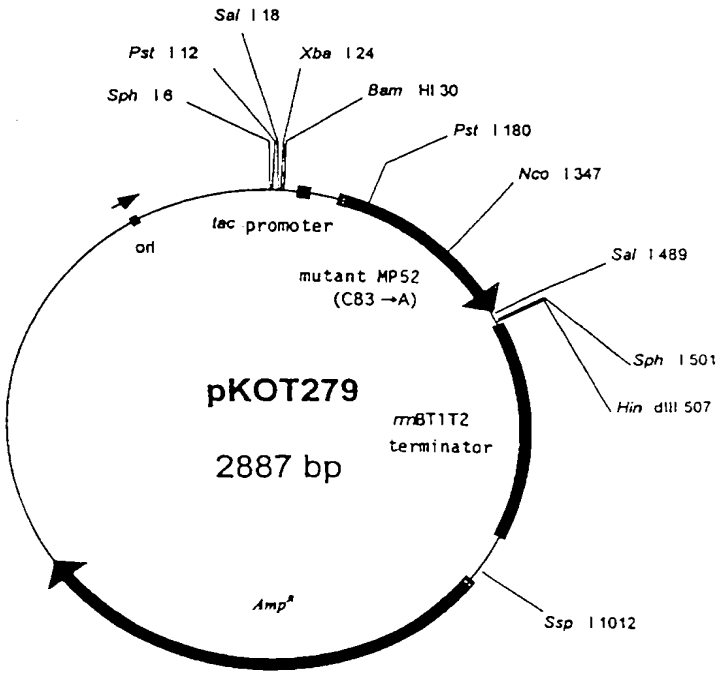
(54) Title: MONOMER PROTEIN WITH BONE MORPHOGENETIC ACTIVITY AND MEDICINAL AGENT CONTAINING THE SAME FOR PREVENTING AND TREATING DISEASES OF CARTILAGE AND BONE

(57) Abstract

The purpose is to provide a monomer protein effective for prevention and therapeutic treatment of bone and/or cartilage diseases. Said purpose is achieved by a monomer protein having an amino acid sequence of which cysteine contributing to dimer formation of a protein belonging to TGF–β superfamily has been replaced with another amino acid. In comparison with the corresponding dimer protein, the monomer protein has a two–fold higher activity in an osteoblast cell line to induce differentiation. Other amino acids are exemplified by serine, threonine, alanine, and valine, and preferably alanine. Said protein is prepared by using *Escherichia coli*, yeast, insect cells, and mammal cells that have been transformed by a plasmid having a DNA sequence capable of expression of said monomer protein.



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MONOMER PROTEIN WITH BONE MORPHOGENETIC ACTIVITY AND MEDICINAL AGENT CONTAINING THE SAME FOR PREVENTING AND TREATING DISEASES OF CARTILAGE AND BONE

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to a monomer protein having an amino acid sequence belonging to TGF-β superfamily, of which cysteine related to a dimer formation of a protein has been replaced with another amino acid. Moreover, the present invention relates to a method for preparing said monomer protein in a large amount and with a high purity by using *Escherichia coli* transformed with a plasmid containing a DNA sequence that can express said monomer protein. Furthermore, the present invention relates to an agent containing said monomer protein for preventing and treating a disease affecting bone and/or cartilage.

(2) Description of the Related Art

Currently, there are known estrogen, calcitonin, vitamin D3, its derivatives and derivatives of bisphosphonic acid as preventive or therapeutic agents for bone diseases. Recently, it has been reported that a bone morphogenetic activity is found in a series of a bone morphogenetic protein (hereinafter referred to as "BMP") belonging to TGF-β superfamily, from BMP-2 to BMP-14.

Moreover, it has been reported that a protein named GDF-5 or human MP52 has a bone morphogenetic activity (WO93/16099, WO95/04819, WO94/15949 and Nature Vol. 368, 1994, p. 639-643). It is considered that mature human MP52 is a protein having 120 amino acid residues starting with alanine at an N-terminal, and its amino acid sequence has been described in these patent applications.

These proteins exist as a homodimer having a single disulfide bond in nature. On the contrary, the manufacture of their recombinant protein is carried out using their homodimers or heterodimers to yield a protein showing the activity. For example, human MP52 has been reported in the publication of unexamined application, JP 031098/97.

Meanwhile, there are two types named type I receptor and type II receptor in the receptors of TGF-β superfamily. Intercellular signal transmission via receptors of TGF-β superfamily containing these bone morphogenetic proteins (dimers) requires simultaneous combination of these proteins to both type I and type II receptors, and it is considered that a polymer is formed by gathering of two or more dimers to do intercellular signal transmission (Bone, Vol. 19, 1996, p. 569-574). It has been considered that for polymer formation it is important that the protein should be a dimer. The activity in a monomer has not yet been found. Moreover, preparation for these monomer recombinants has not yet been carried out.

SUMMARY OF THE INVENTION

The present inventors have attempted a mass production of human MP52 monomers by a genetic engineering technology using *Escherichia coli*. Namely, the present inventors constructed a plasmid of DNA sequence encoding the amino acid sequence having 119 residues described in SEQ ID NO: 1 of the Sequence Listing, among which the codon of the cysteine residue of No. 83, that is related to a disulfide bond between MP52 monomer molecules, was converted to the codon of alanine. In addition, the inventors have succeeded in expressing a large amount of human MP52 monomers using *Escherichia coli* by using the plasmid and refolding to produce monomers of the protein described in SEQ ID NO: 1 of the Sequence Listing with a high purity and a very high yield.

It has been surprisingly found that the monomer has the activity to induce differentiation to osteocytes in some cell lines (MC3T3-E1 and ATDC5) despite that in conventional understanding, only a dimer has a bone morphogenetic activity. The present invention has been completed by observing that the activity to induce differentiation is two-fold higher than that of the dimer on the basis of weight concentration.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a plasmid map of the expression vector

(pKOT279) obtained in Example 1 (2).

Fig. 2 is a comparative figure of osteoblast differentiation promoting activities between the monomer of the present invention and human MP52 dimer. (A) shows the activity in MC3T3-E1 cells and (B) shows that in ATDC5 cells. The white circle shows the activity of the monomer and the black circle shows that of human MP52 dimer.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Namely, the present invention relates to a monomer protein having an amino acid sequence belonging to TGF-β superfamily, of which cysteine related to a dimer formation of the protein has been replaced with another amino acid, a method for expressing said monomer protein, and an agent for preventing and treating a disease affecting bone and/or cartilage containing one or more than one said monomer proteins.

The present invention relates to a monomer protein having an amino acid sequence belonging to TGF-β superfamily, of which cysteine related to a dimer formation of the protein has been replaced with another amino acid. The TGF-β superfamily of the present invention means BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-12, BMP-13, BMP-14, human MP52, GDF-5, GDF-6, GDF-7, etc. Another amino acid may be any amino acid selected from a group consisting of alanine, threonine, serine and valine in consideration of the size of an amino acid side chain. The most preferable amino acid is alanine.

The present invention relates to a monomer protein having an amino acid sequence described in SEQ ID NO.: 1 of the Sequence Listing. In detail, the monomer protein is a protein in which cysteine is replaced with alanine, and the aforesaid cysteine contributes to intermolecular disulfide bond of a human MP52 dimer having an intermolecular disulfide bond, and is present at the 83rd position of the amino acid sequence of SEQ ID NO.: 1 of the Sequence Listing. The monomer protein obtained by the present invention shows a two-fold higher activity in inducing differentiation than a dimer protein made from the monomer protein.

Furthermore, the present invention relates to a method for preparation of said monomer protein to express by using *Escherichia coli*, yeast, insect cells, and mammal cells that have been transformed by a plasmid having a DNA sequence capable of expression of said monomer protein. In detail, the present invention relates to a method for preparation of a protein having 119 amino acid residues derived from human MP52 represented by SEQ ID NO.: 2 of the Sequence Listing, by employing *Escherichia coli*. In other words, the present invention relates to construction of a plasmid having a DNA sequence that encodes an amino acid sequence in which methionine is added to the N-terminal of the amino acid sequence derived from human MP52 in which alanine has replaced cysteine of the 83rd position from 119 residues represented by SEQ ID NO.: 1 of the Sequence Listing. For human MP52 cDNA, a mature portion was solely amplified by polymerase chain reaction (PCR method) by using a plasmid vector as a template DNA containing cDNA described in WO93/16099. The PCR method used in the invention means general amplification from a very small amount of a fragment of DNA or RNA of a nucleic acid by the method described in USP 4,683,195.

In the present invention, a mutant monomer protein was obtained by construction of a plasmid having a DNA sequence that encodes an amino acid sequence in which methionine is added to the N-terminal of the amino acid sequence represented by SEQ ID NO.: 1 of the Sequence Listing, by transformation of the plasmid to *Escherichia coli*, by solubilization of the inclusion body obtained by culturing the *Escherichia coli* and by purification. The present invention relates to a method for preparation of the protein by refolding to have an activity and purifying said protein to a monomer protein described in SEQ ID NO.: 2 of the Sequence Listing. Concretely, for the monomer protein of the present invention, MP52 mutant monomer protein was obtained by applying the solubilized inclusion bodies of *Escherichia coli* to a SP-Sepharose FF column (Amersham Pharmacia Biotech) and to Superdex 200 pg column (Amersham Pharmacia Biotech).

Subsequently, the purified monomer protein of the present invention is obtained by refolding and then by passing through a reversed phase HPLC RESOURCE RPC column (Amersham Pharmacia Biotech). The physical and chemical properties of the present monomer protein obtained are analyzed on the basis of data of an N-terminal amino acid sequence, an amino acid composition, and electrophoresis.

The biological properties of the monomer protein of the present invention were evaluated by the activity to induce differentiation of two kinds of osteoblast cell lines of which promoting alkaline phosphatase activity was already found in a human MP52 dimer. In comparison in the weight concentration, the monomer protein of the present invention showed a two-fold higher activity than that of the conventional dimer protein.

The present invention relates to a preventive or therapeutic agent for cartilage and/or bone diseases having amino acid sequence represented by SEQ ID NO.: 2 of the Sequence Listing as an effective ingredient. In detail, the monomer protein of the present invention has an activity to induce differentiation, i.e., an morphogenetic activity for cartilage and bone, and therefore, relates to a preventive or therapeutic agent for osteoporosis, congenital bone and/or cartilage diseases, and osteoarthritis such as joint osteoarthritis and hip joint osteoarthritis, or arthrosteitis, damage of cartilage such as damage of meniscus, regeneration of bone and cartilage deficit caused by injury and tumor dissection, bone and cartilage deficit, fracture, congenital cartilage and/or bone diseases such as achondroplasia, dyschondrogenesis, achondrogenesis, palatoschisis, and dysosteogenesis, and a deficit of root of teeth and a tooth socket.

Furthermore, the protein of the present invention, having bone and cartilage morphogenetic activity, can be used for therapy of bone graft in an aesthetic surgery field. The therapy includes a field of veterinary surgery.

As in systemic administration method, intravenous, intramuscular, and intra-abdominal administrations are

possible; in an intravenous administration, an intravenous drip can be applied in addition to a general intravenous injection.

An injection preparation can be, for example, a powder preparation for injection. In the case, one or more kinds of appropriate water-soluble excipient such as mannitol, sucrose, lactose, maltose, glucose, or fructose are added to dissolve in water, divided into vials or ampoules, freeze-dried, and hermetically sealed to make as a product.

For a local administration method, there is a method to cover the surface of a cartilage, bone, or tooth of the site with the present protein by using collagen paste, fibrin glue, or other adhesives. Among them, a bone used for bone graft can be also applied to an artificial bone conventionally used as well as a natural bone. The artificial bones include bones made of natural materials or artificial inorganic materials such as metals, ceramics, and glasses. The artificial inorganic materials are preferably exemplified by hydroxyapatite. For example, a metal is used for an internal material and hydroxyapatite for an external material of an artificial bone. Furthermore, the present protein can be administered to a carcinomatous tissue to enhance reconstruction of a bone. It is also possible to use for cartilage grafting.

An administrative dose is determined by a physician in charge in consideration of the following various factors affecting the action of the present protein: the weight of bone and cartilage to reconstruct, the site and condition of the damage of bone and cartilage, sex and age of a patient, severity of the infection, administration duration, and other clinical factors. The dose can vary according to the kind of a carrier used for reconstruction that is realized in combination with the present protein. In general, concerning the dose, ca. $10\text{-}10^6$ ng as the present monomer protein for a given wet weight of a bone and cartilage in the use as a composition containing a carrier and $0.1\text{-}10^4$ μg for one patient as an injection for local and in systemic application are preferably administered in the frequency ranging from

once a day to once a week.

A multiplier effect can be expected by simultaneous application of a known growth factor such as insulin-like growth factor-I for regeneration of a bone and cartilage.

Thus, a monomer made by substitution of cysteine of a protein belonging to TGF-β superfamily and industrial manufacture for a monomer have not been reported. The monomer has a morphogenetic activity for cartilage and bone and is useful as a therapeutic agent for cartilage and/or bone diseases. Furthermore, the monomer protein of the present invention shows a two-fold higher activity per weight than that of a dimer of the protein and allows a half reduction of an effective dose of a therapeutic agent for cartilage and/or bone diseases. This fact can be applied to manufacture of before-mentioned bone morphogenetic factors belonging to TGF-β superfamily.

The monomer protein derived from human MP52 and having an amino acid sequence described in SEQ ID NO.: 2 of the Sequence Listing has a two-fold higher activity in a osteoblast cell line to induce differentiation than that of the dimer and useful as a preventive or therapeutic agent for cartilage and/or bone diseases. Furthermore, a change of an amino acid of the monomer protein of the present invention reduces cysteine and thus, it makes easy preparation of a mass and pure monomer protein possible by using *Escherichia coli*.

EXAMPLES

This invention shall be more illustratively explained by way of the following Examples. The following Examples are to be considered in all respects as illustrative and not restrictive.

Example 1 Preparation of a human MP52 monomer expression vector

(1) Isolation of a mature region of a human MP52 mutant

The human MP52 monomer was prepared by replacing cysteine residue which is regarded as forming a dimer with another amino acid residue in order to prevent the formation of a dimer with the human MP52 monomer. In the present

invention, the codon of cysteine (TGC) of the 83rd of the mature human MP52 starting with proline described in SEQ ID NO.: 1 of the Sequence Listing of WO 96/33215 was converted to the codon of alanine (GCC).

The substitution of an amino acid residue was carried out by using a PCR primer (forward direction) in which an objective mutation has been introduced with reference to the mutation method (Section 8.5) by polymerase chain reaction (PCR) described in Current Protocols in Molecular Biology (John Wiley & Sons, Inc.). The sequence of the PCR primer used was described in SEQ ID NO.: 3 as a sense primer and in SEQ ID NO.: 4 as a reverse primer.

PCR was performed by using a human MP52 expression vector (pKOT245) described in WO96/33215 as a template DNA (10 ng), each 10 pM sense primers and reverse primers, dNTP of 0.4 mM, $MgCl_2$ of 2.5 mM, and LA Taq DNA polymerase (5U, Takara Shuzo Co., Ltd; catalog No. RR013A) in the same test tube. The 30 cycles of reaction was operated of which one cycle included denaturation (94°C, 1 min), primer annealing (55°C, 1 min), and primer elongation (72°C, 2 min). The PCR product was digested by restriction enzymes *Nco*I and *Hin*dIII, separated by electrophoresis with 1.5% low melting point agarose (FMC BioProducts Co., catalog No. 5170B) and purified to obtain a DNA fragment having a ca. 170 bases as an objective product.

The human monomer MP52 expression vector (pKOT279) was prepared by replacing a DNA fragment of *Nco*I-*Hin*dIII in which mutation was introduced by aforementioned method with *Nco*I-*Hin*dIII region of a human monomer MP52 expression vector (pKOT277) made by modifying a human monomer MP52 expression vector (pKOT245) described in WO96/33215. Concretely, by preparing the human monomer MP52 expression vector (pKOT277) from which lacZ promoter, that is transcribed in the reverse direction to a MP52 existing in the downstream of the terminator of the human monomer MP52 expression vector (pKOT245) described in WO96/33215, by digesting said MP52 expression vector (pKOT277) by restriction enzymes *Nco*I and *Hin*dIII, separating by electrophoresis in 1.5 % low melting

point agarose (FMC BioProducts Co., cat. No. 5170B) and by purifying, a DNA fragment having 2717 base pairs was obtained for an objective product. The DNA fragment and the DNA fragment of ca. 170 base pairs to which mutation was introduced, were ligated by using DNA Ligation Kit (Takara Shuzo Co., Ltd., catalog No. 6021) to prepare a human monomer MP52 expression vector (pKOT279, 2.9 kb). The vector was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan, in February 5, 1998 (Deposit no. Bikoukenki no. FERM P-16625) and transferred to the International Depository Authority under Budapest Treaty on February 3, 1999 (Deposit No. FERM BP-6637). For the base sequence of the human MP52 monomer expression vector of the present invention, introduction of the objective mutation and correctness of the base sequence (other sequence than that of the site to which a mutation was introduced) of the human MP52 produced were confirmed by using a DNA sequencer (Amersham Pharmacia Biotech, ALF).

(2) Transformation

Transformation was experimented according to rubidium chloride method of Kushner et al. (Genetic Engineering p. 17, Elsevier. 1978). Namely, pKOT279 was introduced to *Escherichia coli* W3110M according to above method to make the *Escherichia coli* to express a protein in the present invention.

Example 2 Cultivation

(1) Cultivation

The *Escherichia coli* to express a protein of the present invention was precultured in a modified SOC culture medium (Bacto tryptone 20 g/L, Bacto yeast extract 5 g/L, NaCl 0.5 g/L, $MgCl_2$ 0.95 g/L, and glucose 3.6 g/L), 100 mL of cell suspension (Bacto tryptone 20 g/L, citric acid 4.3 g/L, K_2HPO_4 4.675 g/L, KH_2PO_4 1.275 g/L, NaCl 0.865 g/L, $FeSO_4.7H_2O$ 100 mg/L, $CuSO_4.5H_2O$ 1 mg/L, $MnSO_4.nH_2O$ 0.5 mg/L, $CaCl_2.2H_2O$ 2 mg/L, $Na_2B_4O_7.10H_2O$ 0.225 mg/L, $(NH_4)_6Mo_7O_{24}$ 0.1 mg/L, $ZnSO_4.7H_2O$ 2.25 mg/L, $CoCl_2.6H_2O$ 6 mg/L, $MgSO_4.7H_2O$ 2.2 g/L,

thiamine HCl 5.0 mg/L, methionine 2 g/L, and glucose 3 g/L) was added to 5 L of a culture medium for production to culture in a 10 L culture vessel with aerated stirring, isopropyl-β-D-thiogalactopyranoside of 1 mM concentration in a stage reached a logarithmic multiplication prophase (OD_{550}=50) was added to culture by OD_{550} beyond 150. In the culture, the temperature was regulated to 31°C and the pH was regulated to 7.2 by adding ammonia. Dissolved oxygen concentration was regulated to 50% of air saturation by increasing stirring speed in order to prevent decrease in dissolved oxygen concentration. A 50% glucose solution containing 0.1 M phosphate was added to make glucose concentration 0.2% with reference to rapid rise of dissolved oxygen concentration as an indication in order to make a higher cell concentration in culture.

(2) Preparation of the inclusion bodies from *Escherichia coli*

The culture solution obtained by said method was passed three times through a high pressure homogenizer (LAB40-10RBF1, APV, Gohrin Co.) under 560 bar pressure to break cells and centrifuge to collect a precipitate containing the inclusion bodies.

Example 3 Purification

(1) Solubilization of the inclusion bodies from *Escherichia coli*

The inclusion bodies collected were washed twice with 20 mM Tris-HCl buffer solution (pH 8.3) containing 1 M urea and 5 mM EDTA and centrifuged at 4°C and 3,000 x g for 30 min; the precipitate obtained was solubilized by sonication in 20 mM Tris-HCl buffer solution (pH 8.3) containing 8 M urea, 50 mM NaCl, 64 mM DTT, and 5 mM EDTA.

(2) Purification of denatured monomer protein

The solubilized solution was centrifuged at 4°C and 20,000 x g for 30 min and the supernatant was collected. The supernatant collected was applied to SP-Sepharose FF (Amersham Pharmacia Biotech) column equilibrated with 20 mM Tris-HCl buffer solution (pH 8.3), 6 M urea, 10 mM DTT, and 1 mM EDTA, washed with the solution, and eluted with the

solution containing 0.4 M NaCl. The eluate was subjected to gel filtration with a Superdex 200 pg column (Amersham Pharmacia Biotech) equilibrated by 20 mM Tris-HCl buffer solution (pH 8.3), 6 M urea, 0.5 M NaCl, 10 mM DTT, and 1 mM EDTA to obtain a single denatured monomer protein.

(3) Refolding

50 mM Na-Glycine buffer solution (pH 9.8), 0.5 M NaCl, 20 mM CHAPS, and 3 mM GSSG (oxidized glutathione) of nine times quantity were added to the solution of the denatured monomer protein obtained by above treatment followed by stirring to refold at 4°C for 20 h.

(4) Purification of a monomer protein having an activity.

The sample refolded was diluted 2.8 times with 14 mM NaH_2PO_4 and subjected to isoelectric precipitation. The precipitate was collected by centrifugation at 3,000 X g for 20 min and dissolved in 0.05% TFA. The solution was applied to a RESOURSE RPC column (Amersham Pharmacia Biotech) of reverse-phase HPLC previously equilibrated with 0.05% TFA and eluted with 0.05% TFA and 0 - 50% acetonitrile gradient. The eluate was monitored by an absorptiometer at 280 nm absorbancy to obtain a fraction of purified monomer protein of the present invention. To the protein fraction, 5 N NaOH was added to make in the range of between pH 6.5 and 7.5 for precipitation in isoelectric point. The precipitate was collected by centrifugation of 10,000 X g for 10 h and dissolved in 10 mM HCl to make ca. 3 mg/mL to obtain a monomer protein having an activity of the present invention.

(i) N-terminal sequence analysis

The N-terminal analysis of the amino acid composition of the purified monomer protein of the present invention obtained above was carried out by using a sequencer (Applied Biosystem, Model 476A).

(ii) Amino acid composition analysis

The amino acid composition of the purified monomer protein of the present invention obtained above was examined by an amino acid analyzer (Waters, PICO. TAG. WORK STATION).

(iii) Electrophoretic analysis

The molecular weight of the purified monomer protein of

the present invention obtained above was investigated by SDS-PAGE under a non-reduced condition to be a molecular weight of ca. 1.4 kDa.

As the results given by (i), (ii), and (iii), it has been found that the monomer protein of the present invention is a monomer protein having 119 amino acid residues of which N-terminal starts with Pro shown in SEQ ID NO.: 2 of the Sequence Listing.

Example 4 Measurement of biological activity

A differentiation inducing activity was evaluated by employing two cultured cell lines; ATDC5 (Riken Gene Bank, RCB 0565) to differentiate like a cartilage cell derived from a mouse embryonic cell and MC3T3-E1 (Riken Gene Bank, RCB 1126) having properties like those of an osteoblast derived from a mouse, on the basis of reference to alkaline phosphatase promoting activity of said protein. The result is shown in Fig. 2.

ATDC5 and MC3T3-E1 of the concentration of 10,000 cells per 1 mL were suspended in DF culture medium (Gibco Ltd.) containing 5% bovine fetus serum and in MEM-α^- medium (Gibco Ltd.) containing 10% bovine fetus serum, respectively, and inoculated in 24 plates at 1 mL per 1 well to culture at 37°C for 3 days under 5% CO_2.

Subsequently, the cells were rinsed with the MEM-α^- medium without serum, a natural dimer or a monomer protein diluted gradationally with the MEM-α^- medium containing 0.3% bovine albumin was added 0.5 mL per 1 well to start induction of differentiation. The cultivation was carried out for 3 days, the cells were rinsed with PBS (20 mM phosphate buffer solution, 150 mM NaCl, pH 7.4) twice and 250 μL of cytolytic solution (0.2% NP-40, 1 mM $MgCl_2$) was added and kept standing at 37°C for 2 hours. Following this step, the total volume of the cytolytic solution containing cells broken was transferred to a micro tube and centrifuged (10,000 X g, 5 min) to use its supernatant for assay.

An enzyme activity was measured by observing the rise of absorbancy of p-nitrophenol (pNp) being the dissociated product derived from p-nitrophenyl phosphate as the substrate

of the final concentration of 10 mM by dissolving in 0.1 M glycine buffer, pH 10.4, 1 mM $ZuCl_2$, and 1 mM $MgCl_2$, at 405 nm.

The rise of absorbancy was observed every 2 min for 40 min and the alkaline phosphatase promoting activity (μM pNp/min) was calculated on the basis of the data of the range showing linearity.

In addition, the protein concentration of the same supernatant was known by using a BCA Protein Assay Kit (Amersham Pharmacia Biotech) and the alkaline phosphatase activity per protein was represented by nmol pNp/min/mg protein.

Sequence Listing Free Text

<210> 1
<223> Relevant amino acid residues in SEQ ID NO 1 from 1 to 82 and from 84 to 119 in WO 95/04819.
Note : aminoacid residue 83 is alanine instead of cysteine.

<210> 3
<223> Sense PCR primer for mutation introducing.

<210> 4
<223> Reverse PCR primer for mutation introducing.

What is claimed is:

1. A monomer protein comprising an amino acid sequence belonging to TGF-β superfamily, of which cysteine related to a dimer formation of the protein has been replaced with another amino acid.

2. The monomer protein according to claim 1, wherein another amino acid is an amino acid selected from the group consisting of serine, threonine, alanine and valine.

3. The monomer protein according to claim 1 or 2, wherein another amino acid is alanine.

4. A monomer protein comprising an amino acid sequence described in SEQ ID NO.: 2 of the Sequence Listing.

5. A method for expression by using *Escherichia coli*, a yeast, an insect cell, or a mammal cell transformed with a plasmid comprising a DNA sequence that can express a monomer protein according to any one of claims 1 to 4.

6. An agent comprising the monomer protein according to any one of claims 1 to 4 containing an effective amount of the monomer protein for preventing and treating a disease affecting bone and/or cartilage.

7. The agent for preventing and treating a disease affecting bone and/or cartilage according to claim 6, wherein the disease is osteoporosis.

8. The agent for preventing and treating a disease affecting bone and/or cartilage according to claim 6, wherein the disease is osteoarthritis or arthrosteitis.

9. The agent for preventing and treating a disease affecting bone and/or cartilage according to claim 6, wherein the disease is bone fracture.

10. The agent for preventing and treating a disease affecting bone and/or cartilage according to claim 6, wherein the disease is a lack of root of teeth and a tooth socket.

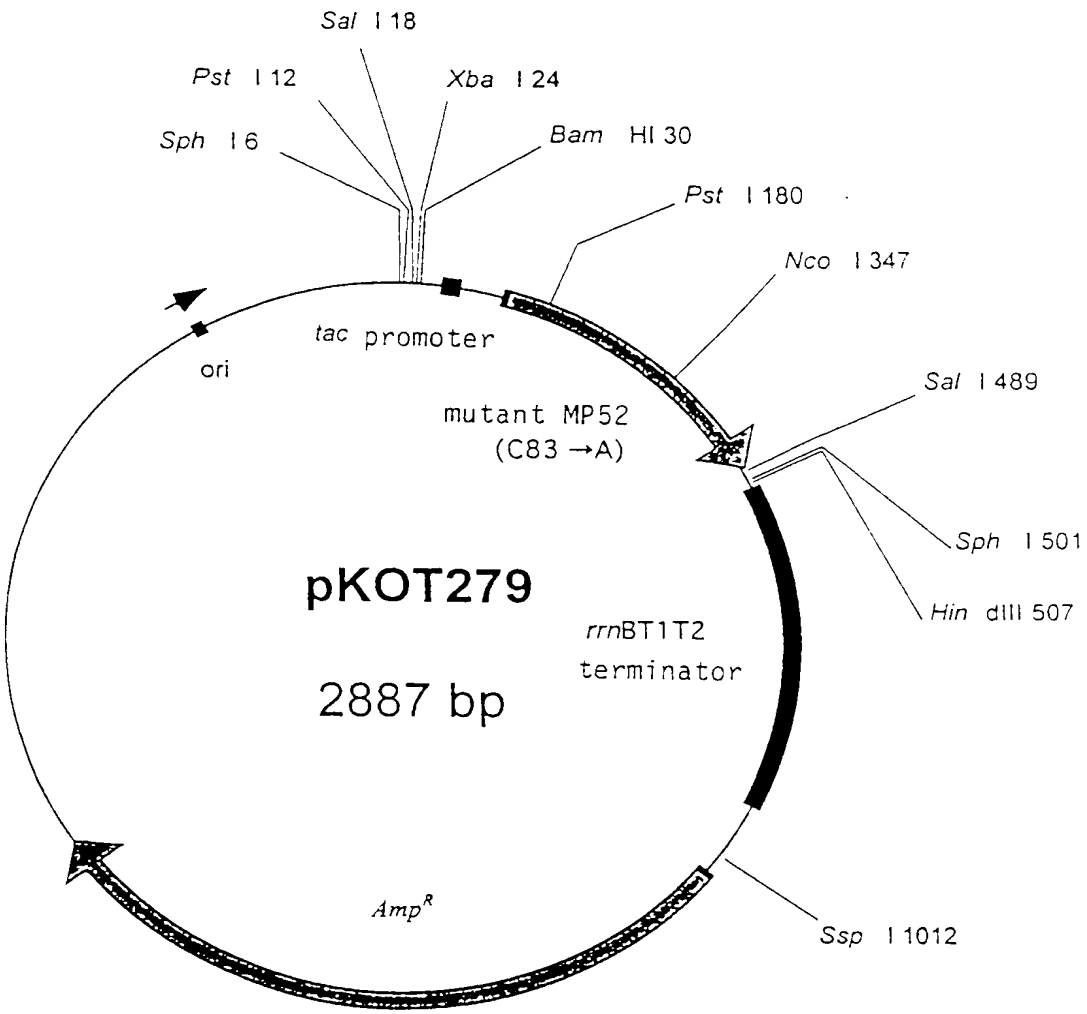
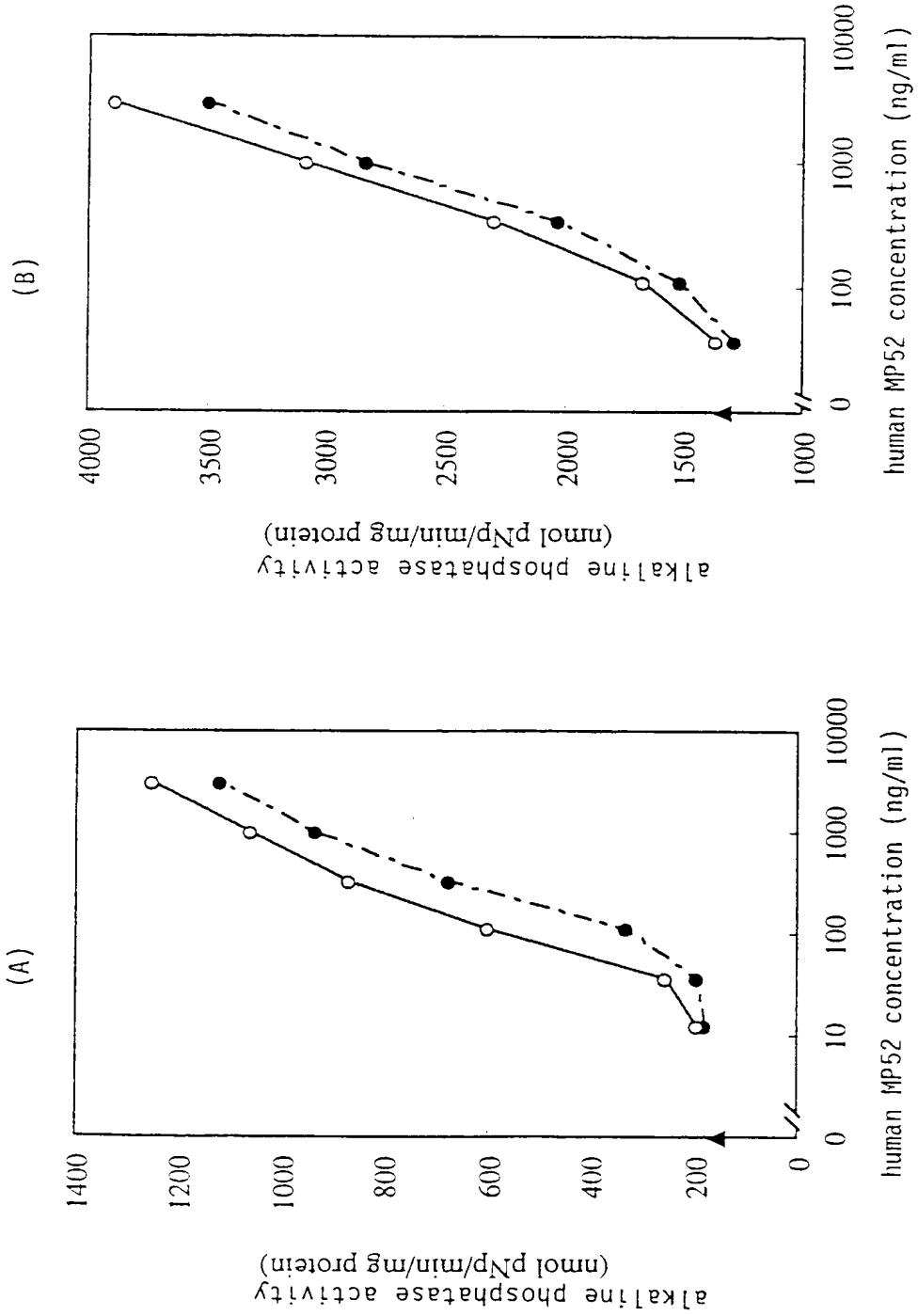

Sal I 18
Pst I 12
Xba I 24
Sph I 6
Bam HI 30
Pst I 180
Nco I 347
Sal I 489
tac promoter
ori
mutant MP52
(C83 →A)
pKOT279
2887 bp
rrnBT1T2
terminator
Sph I 501
Hin dIII 507
Amp^R
Ssp I 1012

FIGURE 1


(A)
alkaline phosphatase activity
(nmol pNp/min/mg protein)
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1200
1000
800
600
400
200
0
0
10
100
1000
10000
human MP52 concentration (ng/ml)
(B)
alkaline phosphatase activity
(nmol pNp/min/mg protein)
4000
3500
3000
2500
2000
1500
1000
0
100
1000
10000
human MP52 concentration (ng/ml)

FIGURE 2

SEQUENCE LISTING

<110> Hoechst Marion Roussel

<120> Novel monomer protein with bone morphogenetic activity and medicinal agent containing the same for preventing and treating diseases of cartilage and bone.

<130> JH98K008 PCT SEQUENCES IN ENGLISH

<140>
<141>

<150> 10-141379
<151> 1998-05-22

<160> 4

<170> PatentIn Ver. 2.1

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Note : aminoacid residue 83 is alanine instead of cysteine.

<300>
<301> HOTTEN, Gertrud
NEIDHARDT, Helge
PAULISTA, Michael

<302> New growth/differentiation factor of the tgf-beta familie.

<310> WO 95/04819

<311> 1995-02-16

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cgc tgc agt cgg aag gca ctg cat gtc aac ttc aag gac atg ggc tgg   96
Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp
             20                  25                  30

gac gac tgg atc atc gca ccc ctt gag tac gag gct ttc cac tgc gag   144
Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu
         35                  40                  45

ggg ctg tgc gag ttc cca ttg cgc tcc cac ctg gag ccc acg aat cat   192
Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
     50                  55                  60

gca gtc atc cag acc ctg atg aac tcc atg gac ccc gag tcc aca cca   240
Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro
 65                  70                  75                  80

ccc acc gcc tgt gtg ccc acg cga ctg agt ccc atc agc atc ctc ttc   288
Pro Thr Ala Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe
                 85                  90                  95

att gac tct gcc aac aac gtg gtg tat aag cag tat gag gac atg gtc   336
Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val
            100                 105                 110

gtg gag tcg tgt ggc tgt agg                                     357
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             20                  25                  30

Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu
         35                  40                  45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
     50                  55                  60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro
 65                  70                  75                  80

Pro Thr Ala Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe
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Val Glu Ser Cys Gly Cys Arg
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<223> Reverse PCR primer for mutation introducing.

<400> 4

cccaagcttg catgcctgcc ggtcgactac ctacagc 37

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/IB 99/00866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/51 C12P21/02 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	MASON A J : "FUNCTIONAL-ANALYSIS OF THE CYSTEINE RESIDUES OF ACTIVIN-A" MOLECULAR ENDOCRINOLOGY, (MAR 1994) VOL. 8, NO. 3, PP. 325-332. ISSN: 0888-8809., XP002111994	1-3,5
Y	abstract page 329, paragraph 3 - page 330, paragraph 1	6-10

X	AMATAYAKUL-CHANTLER ET AL.: "'Ser77!Transforming growth factor-beta1" J. BIOL. CHEM., vol. 269, no. 44, 4 November 1994 (1994-11-04), pages 27687-27691, XP002111995	1,2,5
Y	the whole document	6-10

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 1999	30/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/00866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	HÜSKEN-HINDI ET AL.: "Monomeric activin A retains high receptor binding affinity but exhibits low biological activity" J. BIOL. CHEM., vol. 269, no. 30, 29 July 1994 (1994-07-29), pages 19380-19384, XP002111996	1,2,5
Y	the whole document	6-10
Y	WO 92 19262 A (CELTRIX PHARMA) 12 November 1992 (1992-11-12) the whole document page 5, line 24-31 page 9, line 14-23 page 13, line 18-33 claims 1-3,15-17,27-36	6-10
Y	WO 92 14481 A (CELTRIX PHARMA) 3 September 1992 (1992-09-03) the whole document claims 1,10,11,13,15,17 examples 3-5	6-10
Y	US 5 158 934 A (AMMANN ARTHUR J ET AL) 27 October 1992 (1992-10-27) the whole document claims 1-4,6 examples 1-3	6-10
A	WO 97 04095 A (MATSUMOTO TOMOAKI ;HOECHST JAPAN (JP); KIMURA MICHIO (JP); FUJINO) 6 February 1997 (1997-02-06) abstract	4,6-10
P,A	& EP 0 866 125 A (HOECHST MARION ROUSSEL LTD) 23 September 1998 (1998-09-23) the whole document	4,6-10
A	MCDONALD ET AL.: "A structural superfamily of growth factors containing a cystine knot motif" CELL, vol. 73, 7 May 1993 (1993-05-07), pages 421-424, XP002111999 the whole document	1-4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/IB 99/00866

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9219262 A	12-11-1992	US 5118667 A	02-06-1992
		AU 660182 B	15-06-1995
		AU 1891392 A	21-12-1992
		CA 2102429 A	04-11-1992
		EP 0514720 A	25-11-1992
		JP 2831132 B	02-12-1998
		JP 6511233 T	15-12-1994
WO 9214481 A	03-09-1992	US 5208219 A	04-05-1993
		AU 1460192 A	15-09-1992
		US 5413989 A	09-05-1995
US 5158934 A	27-10-1992	US 5409896 A	25-04-1995
		US 5422340 A	06-06-1995
		US 5604204 A	18-02-1997
WO 9704095 A	06-02-1997	JP 9031098 A	04-02-1997
		AU 704364 B	22-04-1999
		AU 6530496 A	18-02-1997
		CA 2224289 A	06-02-1997
		CN 1196087 A	14-10-1998
		EP 0866125 A	23-09-1998
		NO 980300 A	23-01-1998

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (8-96)

Approved for use through 9/30/98. OMB 0651-0032

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	146.1358
First Named Inventor	S. KAWAI et al
COMPLETE IF KNOWN	
Application Number	PCT/IB99/00866
Filing Date	May 14, 1999
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MONOMER PROTEIN WITH BONE MORPHOGENETIC ACTIVITY AND MEDICINAL AGENT CONTAINING THE SAME FOR PREVENTING AND TREATING DISEASES OF CARTILAGE AND BONE

(Title of the Invention)

the specification of which

[ ] is attached hereto

OR

[X] was filed on (MM/DD/YYYY) May 14, 1999 as United States Application Number or PCT International Application Number PCT/IB99/00866 and was amended on (MM/DD/YYYY) _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56

I hereby claim foreign priority benefits under Title 35 United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	Certified Copy Attached? NO
10/141379	Japan	5/22/98	[ ]	[ ]	[ ]
PCT/IB99/00866	IB	5/14/99	[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
		[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

146.1358

Please type a plus sign (+) inside this box +

PTO/SB/81 (8-98)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Charles A. Muserlian	19,683		
Jordan B. Bierman	18,629		
Donald C. Lucas	31,275		
Bierman, Muserlian and Lucas	18,818		

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

Direct all correspondence to:

Name	Bierman, Muserlian and Lucas				
Address					
Address	600 Third Avenue				
City	New York	State	New York	ZIP	10016
Country	U.S.A.	Telephone	(212) 661-8000	Fax	(212) 661-8002

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name	SHINJI	Middle Initial		Family Name	KAWAI	Suffix e.g. Jr.	
Inventor's Signature					Date		
Residence: City	Paris	State		Country	France	Citizenship	FR
Post Office Address							
Post Office Address	416,rue Emile Dubois						
City	Paris	State		Zip	F-75014	Country	France

☒ Additional inventors are being named on supplemental sheet(s) attached hereto

146.1358

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PTO/SB/01 (8-96)

Under the Paperwork Reduction Act of [redacted] persons are required to respond to a collection of information unless it contains a valid OMB control number.

Patent and Trad[redacted] for use through 9/30/98 OMB 0651-0032
Office. U.S. DEPARTMENT OF COMMERCE

DECLARATION

ADDITIONAL INVENTOR(S) Supplemental Sheet

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name	MICHIO	Middle Initial		Family Name	KIMURA	Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City	Kanagawa	State		Country	Japan	Citizenship	Japan
Post Office Address							
Post Office Address	9-8-304, Tsurugadai, Chigasaki-shi						
City	Kanagawa	State		Zip	253-0003	Country	Japan

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name	YOSHIFUMI	Middle Initial		Family Name	MURAKI	Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City	Tokyo	State		Country	Japan	Citizenship	Japan
Post Office Address	Hoechst Marion Roussel Ltr. Product RealizationDept.						
Post Office Address	17-51, Akasaka, 2-chome, Minato-ku,						
City	Tokyo	State		Zip	107-8465	Country	Japan

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name	MIEKO	Middle Initial		Family Name	KATSUURA	Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City	Tokyo	State		Country	Japan	Citizenship	Japan
Post Office Address							
Post Office Address	2-14-2-106 Sakae-cho, Higashimurayama-shi,						
City	Tokyo	State		Zip	189-0013	Country	Japan

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name		Middle Initial		Family Name		Suffix e.g. Jr.	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip		Country	

☐ Additional inventors are being named on supplemental sheet(s) attached hereto

(January 1997)

16

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
JH98K008/PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB99/00866	14/05/1999	22/05/1998
International Patent Classification (IPC) or national classification and IPC C12N15/12		
Applicant HOECHST MARION ROUSSEL LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/11/1999	01.08.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel +49 89 2399 - 0 Tx: 523656 epmu d Fax +49 89 2399 - 4465	Authorized officer van Heusden, M Telephone No. +49 89 2399 8145

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/00866

I. Basis of th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-14 as originally filed

Claims, No.:

1-10 as originally filed

Drawings, sheets:

1/2-2/2 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4, 6-10
	No:	Claims	1-3, 5
Inventive step (IS)	Yes:	Claims	4
	No:	Claims	1-3, 5-10
Industrial applicability (IA)	Yes:	Claims	1-10
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Additional remarks to section V:

1. Citations

1.1 The documents mentioned in this IPER are numbered as in the International Search Report (ISR), i.e. D1 corresponds to the first document of the ISR etc.

2. Novelty (Article 33(2) PCT)

2.1 The present application discloses a monomeric protein comprising an amino acid sequence belonging to the TGF-β superfamily, wherein the cysteine residue related to dimer formation has been replaced with another amino acid. More particularly it relates to said monomeric protein being the human MP52 protein containing said amino acid replacement. It further relates to a method to express said protein recombinantly and to an agent comprising said monomeric protein for preventing and treating a disease affecting bone and/or cartilage.

2.2 The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject matter of claims 1-3 and 5 is not novel in view of documents D1-D3.

2.3 Document D1 discloses the recombinant expression of activin A (a member of the TGF-β superfamily) in which Cys-80 (involved in dimer formation) is replaced by Ala (p. 330, right column, section 'construction of pActA and pcys mutants' and Figure 3). Thus D1 anticipates the subject matter of claims 1-3 and 5.

2.4 Document D2 discloses the recombinant expression of TGF-β1 in which Cys-77 (involved in dimer formation) is replaced by Ser (p. 27687, section 'Synthesis and Purification of [Ser77]TGF-β1, and p. 27688, Figure 2). Thus D2 anticipates the subject matter of claims 1-2 and 5.

2.5 Document D3 discloses the recombinant expression of activin A in which Cys-80 (involved in dimer formation) is replaced by Ser (p. 19382, Figure 1). Thus D3 anticipates the subject matter of claims 1-2 and 5.

3. Inventive step (Article 33(3) PCT)

3.1 The subject matter of claim 4 is considered novel and inventive because there is no incentive in the prior art to produce a monomeric form of MP-52. In fact the cited prior art (D1-D3) teaches away from producing biologically active monomeric forms of members of the TGF- superfamily, since all three documents show that the monomeric form has little or no biological activity.

3.2 The subject matter of claims 5-10 could be considered inventive only insofar these claims refer to the novel and inventive protein according to claim 4.

4. Industrial applicability (Article 33(4) PCT)

The subject matter of claims 1-10 is industrially applicable.

Additional remarks to section VIII:

The following objections are raised under **Article 6 PCT** concerning the clarity of the claims:

1. Claims 2 and 3 lack clarity in that it isn't clear to what the wording 'another amino acid' refers, in the absence of the word '**said** ....'.

2. The subject matter of claims 6-10 is not enabled: the applicant has shown that monomeric MP-52 having the amino acid sequence of SEQ ID NO:2 has osteoblast differentiation activity, even stronger than the wildtype dimer form of the protein. However, there is no indication in the description that the monomeric form of any member of the TGF-β superfamily will have osteoblast differentiation activity. In fact the cited prior art (D1-D3) shows that the monomeric forms of activin A and of TGF-β1 do not have biological activity (neither receptor binding nor signalling effects). Therefore it is highly unlikely that the medical applications to which claims 6-10 refer are applicable to any member of the TGF-β superfamily.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
JH98K008/PCT.		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 99/ 00866	14/05/1999	22/05/1998
Applicant		
HOECHST MARION ROUSSEL LTD. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

MONOMER PROTEIN WITH BONE MORPHOGENETIC ACTIVITY AND MEDICINAL AGENT CONTAINING THE SAME FOR PREVENTING AND TREATING DISEASES OF CARTILAGE AND BONE

5. With regard to the **abstract**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[ ] as suggested by the applicant.

[X] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

The purpose is to provide a monomer protein effective to prevention and therapeutic treatment of bone and/or cartilage diseases.
Said purpose is achieved by a monomer protein having an amino acid sequence of which cysteine contributing to dimer formation of a protein belonging to TGF-beta superfamily has been replaced with another amino acid. In comparison with the corresponding dimer protein, the monomer protein has a two-fold higher activity in an osteoblast cell line to induce differentiation. Other amino acids are exemplified by serine, threonine, alanine, and valine, and preferably alanine. Said protein is prepared by using Escherichia coli, yeast, insect cells, and mammal cells that have been transformed by a plasmid having a DNA sequence capable of expression of said monomer protein.

INTERNATIONAL SEARCH REPORT

[redacted] ernational Application No
[redacted] CT/IB 99/00866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/51 C12P21/02 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MASON A J : "FUNCTIONAL-ANALYSIS OF THE CYSTEINE RESIDUES OF ACTIVIN-A" MOLECULAR ENDOCRINOLOGY, (MAR 1994) VOL. 8, NO. 3, PP. 325-332. ISSN: 0888-8809., XP002111994	1-3,5
Y	abstract page 329, paragraph 3 - page 330, paragraph 1	6-10

X	AMATAYAKUL-CHANTLER ET AL.: "'Ser77!Transforming growth factor-beta1" J. BIOL. CHEM., vol. 269, no. 44, 4 November 1994 (1994-11-04), pages 27687-27691, XP002111995	1,2,5
Y	the whole document	6-10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 1999	30/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

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Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/IB 99/00866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
X	HÜSKEN-HINDI ET AL.: "Monomeric activin A retains high receptor binding affinity but exhibits low biological activity" J. BIOL. CHEM., vol. 269, no. 30, 29 July 1994 (1994-07-29), pages 19380-19384, XP002111996	1,2,5
Y	the whole document	6-10
Y	WO 92 19262 A (CELTRIX PHARMA) 12 November 1992 (1992-11-12) the whole document page 5, line 24-31 page 9, line 14-23 page 13, line 18-33 claims 1-3,15-17,27-36	6-10
Y	WO 92 14481 A (CELTRIX PHARMA) 3 September 1992 (1992-09-03) the whole document claims 1,10,11,13,15,17 examples 3-5	6-10
Y	US 5 158 934 A (AMMANN ARTHUR J ET AL) 27 October 1992 (1992-10-27) the whole document claims 1-4,6 examples 1-3	6-10
A	WO 97 04095 A (MATSUMOTO TOMOAKI ;HOECHST JAPAN (JP); KIMURA MICHIO (JP); FUJINO) 6 February 1997 (1997-02-06) abstract	4,6-10
P,A	& EP 0 866 125 A (HOECHST MARION ROUSSEL LTD) 23 September 1998 (1998-09-23) the whole document	4,6-10
A	MCDONALD ET AL.: "A structural superfamily of growth factors containing a cystine knot motif" CELL, vol. 73, 7 May 1993 (1993-05-07), pages 421-424, XP002111999 the whole document	1-4

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INTERNATIONAL SEARCH REPORT

mation on patent family members

[redacted]ternational Application No
PCT/IB 99/00866

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9219262 A	12-11-1992	US 5118667 A	02-06-1992
		AU 660182 B	15-06-1995
		AU 1891392 A	21-12-1992
		CA 2102429 A	04-11-1992
		EP 0514720 A	25-11-1992
		JP 2831132 B	02-12-1998
		JP 6511233 T	15-12-1994
WO 9214481 A	03-09-1992	US 5208219 A	04-05-1993
		AU 1460192 A	15-09-1992
		US 5413989 A	09-05-1995
US 5158934 A	27-10-1992	US 5409896 A	25-04-1995
		US 5422340 A	06-06-1995
		US 5604204 A	18-02-1997
WO 9704095 A	06-02-1997	JP 9031098 A	04-02-1997
		AU 704364 B	22-04-1999
		AU 6530496 A	18-02-1997
		CA 2224289 A	06-02-1997
		CN 1196087 A	14-10-1998
		EP 0866125 A	23-09-1998
		NO 980300 A	23-01-1998